Dissociation Reactions of Gaseous Ferro-, Ferri-, and Apo-cytochrome *c* Ions

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Electrochemical reduction of the iron bound in the heme group of cytochrome *c* is shown to occur in the nano-electrospray capillary if the protein is sprayed from neutral water using a steel wire as the electrical contact. Quadrupole ion trap collisional activation is used to study the dissociation reactions of cytochrome *c* as a function of the oxidation state of the iron. Oxidized (Fe(III)) cytochrome *c* dissociates via sequence-specific amide bond cleavage, while the reduced (Fe(II)) form of the protein dissociates almost exclusively by loss of protonated heme. Apo-cytochrome *c*, from which the heme has been removed either via gas-phase dissociation of the reduced holo-protein or via solution chemistry, dissociates via amide bond cleavage in similar fashion to the oxidized holo-protein. (J Am Soc Mass Spectrom 2001, 12, 873–876) © 2001 American Society for Mass Spectrometry

Gas-phase dissociation of whole protein ions can provide a means for protein identification and sequence analysis (referred to as the "top-down" approach) [1, 2] without the need for the time-consuming digestion and separation steps commonly used in modern proteomics (the "bottom-up" approach) [3]. However, the body of observations on whole protein dissociation is still relatively small. In particular, the roles of charge state and post-translational modifications on protein dissociation reactions and on the utility of these reactions for protein identification and characterization are still being explored. The dissociation of cytochrome *c*, a 104 residue electron transport protein with an iron-containing heme group covalently bound to the cysteines at positions 14 and 17, has been extensively studied using a wide variety of activation methods [4–7]; however, no study of the effect of the oxidation state of the iron in the heme group on the dissociation of the protein has been reported. We show here that oxidized (Fe(III)) cytochrome *c* dissociates via sequence-specific amide bond cleavage. In contrast, cytochrome *c* which has been reduced to the Fe(II) state, either electrochemically in the nano-electrospray capillary or with appropriate solution-phase chemistries, remains reduced upon nano-electrospray ionization, and the +7 and +8 charge states of the reduced protein dissociate almost exclusively by loss of protonated heme. Previous reports on cytochrome *c* dissociation have attributed the lack of informative fragmentation near the N-terminus to the presence of the heme group in that region [2]; however, MS/MS of apo-cytochrome *c* reveals that this region of the protein is relatively silent with respect to dissociation under ion trap collisional activation conditions even after the heme is removed.

Experimental

Cytochrome *c* (bovine heart) was purchased from Sigma Chemical Co. (St. Louis, MO) and was nano-electrosprayed at a concentration of 10 μM from the specified solutions using drawn borosilicate glass capillaries. Electrical contact to the solution was made by inserting a stainless steel or platinum wire into the solution through the back of the capillary. Ions were introduced into a Finnigan (San Jose, CA) ion trap mass spectrometer (ITMS) through an interface that has been described previously [8]. Individual charge states were isolated and driven to collision-induced dissociation (CID) by application of a resonant frequency across the end caps in the presence of helium bath gas. Typical resonance activation of +7 cytochrome *c* (all forms) used a frequency of 89 kHz (q_z = 0.2) with an amplitude of 160–170 mV_{p-p}, applied for 300 ms with a helium pressure of 1 millitorr. The multiply-charged product ions resulting from CID were subjected to ion/ion proton transfer reactions with singly-charged anions of perfluorodimethylcyclohexane (PDCH) generated and injected into the ion trap as described previously [9]. These reactions reduce the charge states of the product ions largely to +1, removing charge state

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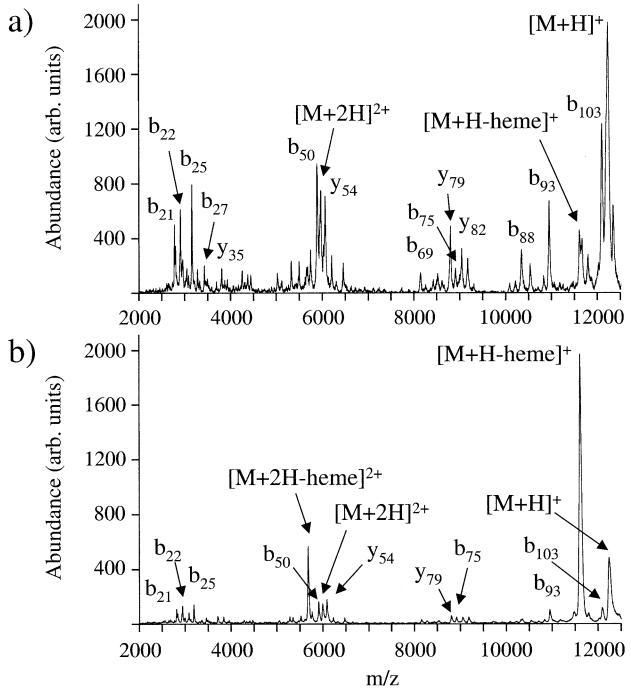

Figure 1. Post ion/ion MS/MS spectra of the +7 ions of cytochrome *c* obtained via nano-electrospray from (**a**) 1% acetic acid and (**b**) pure water, using a stainless steel wire as the electrical contact. Although expressed in arbitrary units, the abundance scales for the two spectra are the same

ambiguity and simplifying interpretation of the relatively low resolution spectra obtained from quadrupole ion trap mass analysis. All MS/MS spectra shown herein are the so-called "post-ion/ion reaction" spectra obtained via these charge-reducing reactions.

Results and Discussion

Figure 1 shows the post-ion/ion reaction CID spectra for the +7 ions of cytochrome *c* electrosprayed from 1% acetic acid (Figure 1a) and pure water (Figure 1b), using a stainless steel wire as the electrical contact. The ions derived from the 1% acetic acid solution show abundant sequence-specific b-and y-type products resulting from amide bond cleavage [10, 11], while the ions from the pure water solution show almost exclusive loss of heme. It was determined from the pre-ion/ion reaction spectrum that the heme was lost as a protonated ion (data not shown). Note that amide bond cleavage channels are also observed for the ions generated from the pure water solution. These products have similar relative abundances to those obtained from the 1% acetic acid solution, but have much lower overall abundance relative to the initial abundance of the precursor ion, which was similar for Figure 1a and b. The change in favored dissociation channels with solution pH may be caused by a change in the protein conformation or the oxidation state of the iron bound in the heme group. Changes in the kinetics of heme loss from holo-myoglobin and holo-hemoglobin α-chain ions have been attributed to changes in protein conformation with solution pH [12]. A number of experiments were conducted to determine why the precursor ions derived from pure water so highly favored the loss of protonated heme.

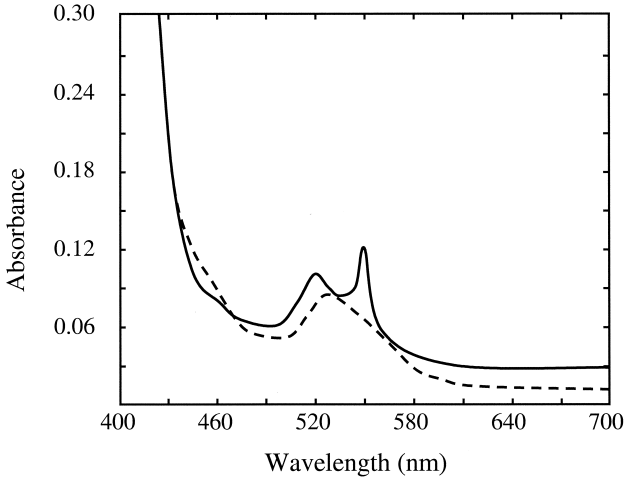

Figure 2. Visible spectra of 10 μM cytochrome *c* in pure water before (dashed line) and after (solid line) nano-electrospray using a stainless steel wire for electrical contact to the solution.

Nano-electrospray of cytochrome *c* from a reducing solution of 1 mM sodium ascorbate in 1% acetic acid yielded +7 ions that also showed abundant protonated heme loss. This result strongly suggests that the observed change in behavior is related to the oxidation state of the iron and not to the protein conformation; however, this conclusion requires that cytochrome *c* sprayed from pure water be reduced electrochemically in the nano-electrospray capillary. Visible spectroscopy was used to confirm that this reduction does occur, as shown in Figure 2 . The dashed trace is the visible spectrum recorded for cytochrome *c* in pure water before spraying, and indicates that the protein was oxidized. The solid trace was recorded by extracting the remaining solution from the nano-electrospray capillary after spraying from a pure water solution. The large absorbance at 550 nm in the solid trace shows unequivocally that the protein was reduced in the capillary. The reduction appears to be related to the use of a stainless steel wire as the electrical contact. When a platinum wire was used instead of a stainless steel wire, the reduction process did not occur, as evidenced by the lack of charged heme loss upon CID of +7 cytochrome *c*, and by visible spectroscopy results obtained by extracting the remaining solution following electrospray. Oxidation of the stainless steel wire during the electrospray process generates Fe(II) ions in solution, which may reduce the protein. Cytochrome *c* can be reduced in solution at room temperature by the addition of ferrous sulfate ($Fe(II)SO_4$) [13]. An aqueous solution of cytochrome *c* containing 100 μM ferrous sulfate was sprayed using a platinum wire and abundant heme loss from +7 cytochrome *c* was observed, supporting the conclusion that the reduction occurs due to the presence of Fe(II) ions.

That cytochrome *c* is reduced in the oxidizing environment of a positive ion electrospray source [14] when sprayed from neutral water is a surprising observation. From the results described above, it can be concluded that reduction in the nano-electrospray capillary occurs due to the electrochemical generation of iron in the Fe(II) state by oxidation of the stainless steel wire used as the electrical contact. When a platinum wire is substituted, no reduction is observed, and no heme loss is observed unless the solution contains a reducing agent such as sodium ascorbate or ferrous sulfate. Reduction of the protein is known to be slower at low pH, [15, 16] explaining why little or no reduction was observed in the 1% acetic acid solution, even when a stainless steel wire was used. Previously, other workers have reported that cytochrome *c* reduced in solution is rapidly re-oxidized during the electrospray process unless oxygen is rigorously excluded from the solution [17, 18], although there is one report that cytochrome *c* cations were kept in the reduced form during electrospray without careful exclusion of oxygen.[5] In the work reported here, it was found that cytochrome *c* reduced in solution could be readily sprayed from solutions containing the reducing agent, hence it was unnecessary to exclude oxygen from the solution.

The reason for the marked difference in the observed dissociation behavior of cytochrome *c* with oxidation state is likely due to the presence or absence of a charge located on the heme group. Intramolecular solvation of the charge present in the oxidized form may cause the heme group to be bound more tightly. Alternatively, the charge may inhibit migration of a proton to effect cleavage of the thioether-heme linkages. As noted above, there are peaks resulting from amide bond cleavage evident in Figure 1b, with similar relative abundances to those observed for the oxidized protein in Figure 1a. This is probably due to competitive dissociation between amide bond cleavage and heme loss in the reduced species. If so, then the reduced form of the protein yields similar sequence information to the oxidized form, albeit with much lower abundances of the informative sequence ions due to the fact that most of the precursor ions dissociate via the heme-loss channel. Note, however, that we cannot rule out the possibility that the amide bond cleavage peaks observed in Figure 1b arise from the presence of some residual oxidized protein in the precursor ion population. Similarly, there is some charged heme loss evident in the spectrum of Figure 1a, suggestive of a small degree of reduction occurring in the 1% acetic acid solution, although again, this may potentially be accounted for by competition within the oxidized species.

The oxidation state of the iron in the heme affects the facility with which heme may be lost from cytochrome *c*. The effect of the presence of heme on the amide bond cleavages observed is also of interest. Shown in Figure 3 is the MS^3 spectrum of +7 apo-cytochrome *c*, generated via MS/MS of +8 cytochrome *c* reduced electrochemically. Reduced +8 cytochrome *c* dissociates similarly to reduced +7 (Figure 1b), showing almost exclusively protonated heme loss. The MS^3 data show that +7 apo-cytochrome *c* dissociates similarly to the oxidized holo-protein (Figure 1a). Interestingly, the "heme-fingerprint" region surrounding cysteines 14 and 17 shows very little dissociation even after the heme is removed. Apo-cytochrome *c* may also be prepared in solution via reaction with silver sulfate [13]; the +7 ions of apo-cytochrome *c* generated in solution dissociated identically to those formed in the gas-phase by MS/MS of reduced +8 (data not shown).

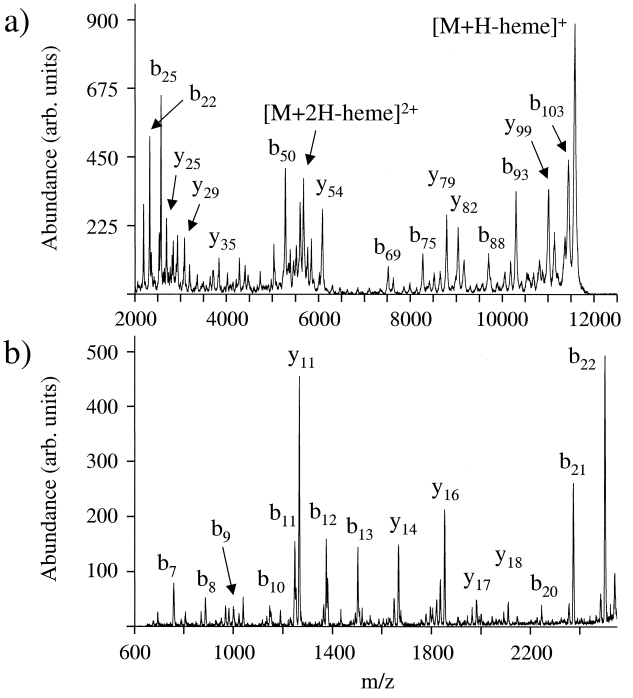

Figure 3. Post ion/ion MS^3 CID spectra of the $[M+8H\text{-heme}^+]^{7+}$ ion of cytochrome *c* acquired at resonance ejection conditions of (**a**) 17000 Hz and 800 mV_{p-p}, and (**b**) 89202 Hz and 3900 mV_{p-p}.

Conclusions

The amide bond cleavage channels reported here for all three forms of +7 cytochrome *c* show that cleavage is favored at the C-terminal of aspartic acid, glutamic acid, and lysine, and at the N-terminal of proline, all channels which have been reported to be favored in protein ion CID [19–21]. A future publication will detail the dissociation behavior of oxidized and reduced cytochrome *c* as a function of charge state, and will address the sequence information obtained for the holo- and apo-protein via quadrupole ion trap CID.

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